Phosphorylation of a 16-kDa protein by diacylglycerol-activated protein kinase C in vitro and by vasopressin in intact hepatocytes

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A 16-kDa protein present in a purified rat liver plasma membrane fraction and also in cytosol can be phosphorylated by endogenous diacylglycerol-activated protein kinase C. In intact hepatocytes prelabeled with ^{32}P, vasopressin causes a rapid increase in the phosphorylation of a 16-kDa protein having a similar p*I* value to that observed in in vitro studies. These findings suggest that vasopressin-induced phosphorylation of the 16-kDa in the intact hepatocyte may reflect increased activity of protein kinase C, secondary to membrane polyphosphoinositide breakdown. Phosphorylation of the 16-kDa protein may thus be part of the coordinated mechanism associated with hormonal regulation of cellular Ca^{2+} fluxes.

Protein phosphorylation *Diacylglycerol-activated protein kinase C* *Vasopressin* *Hepatocyte* *Hormone action*

1. INTRODUCTION

A variety of hormones including α-adrenergic agents, vasopressin and angiotensin II have been shown to increase hepatic glycogenolysis through elevation of cytosolic Ca^{2+} and allosteric activation of phosphorylase kinase (review [1,2]. It appears that the rise in cytosolic Ca^{2+} is caused at least in part by release of calcium from intracellular pools [1–3], but the mechanisms by which this may be coupled with hormone-receptor occupancy are poorly defined. An early event which is common to a variety of cells following activation by hormones is enhanced metabolism of inositol phospholipids [4,5], with polyphosphoinositide metabolism being the focus of recent attention [6]. The ^{32}P content of phosphatidylinositol 4,5-bisphosphate (TPI) is decreased rapidly after treatment of prelabeled hepatocytes with Ca^{2+}-mobilizing hormones [7–9], yielding 1,2-diacylglycerol and inositol 1,4,5-trisphosphate as the initial products of TPI breakdown. On the basis of such studies carried out in this laboratory [9] it has been proposed that changes in the concentration of some allosteric effector of one or more cellular transport systems and/or changes in the phosphorylation state of a regulatory protein(s) elicited by the diacylglycerol-activated protein kinase C [10,11], could provide a mechanism linking TPI breakdown with Ca^{2+} mobilization.

Investigation of the potential significance of protein kinase C in this regard necessitates identification of potential protein substrates for this enzyme in liver. This study indicates that a 16-kDa protein present in a purified rat liver plasma membrane fraction and also in cytosol, can be phosphorylated by endogenous protein kinase C.

Abbreviation: SDS, sodium dodecyl sulfate

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Furthermore, in intact hepatocytes prelabeled with ^{32}P, vasopressin causes a rapid increase in the phosphorylation of a 16-kDa protein having a similar p*I* value to that observed in in vitro studies. These findings suggest that vasopressin-induced phosphorylation of the 16-kDa in the intact hepatocyte may reflect increased activity of protein kinase C, secondary to membrane polyphosphoinositide breakdown.

2. MATERIALS AND METHODS

Carrier-free [^{32}P]orthophosphoric acid and [γ-^{32}P]ATP were purchased from Amersham. Electrophoresis reagents were from BioRad; M_r-markers, arginine vasopressin, 1,2-diolein and phosphatidylserine from Sigma; all other reagents were of analytical grade.

Rat liver plasma membranes were prepared as in [12]. Purity was ascertained using as marker enzymes, 5′-nucleotidase [13], cytochrome oxidase [14], and NADPH cytochrome *c* reductase [15]. Hepatocytes were isolated from fed, male Sprague-Dawley rats (180–220 g) as in [16]. After washing, the cells were resuspended at a final concentration of 5 mg dry wt/ml in a modified Krebs bicarbonate buffer containing 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 15 mM glucose, 0.1% (w/v) dialyzed bovine serum albumin and carrier-free $^{32}P_i$ (1 mCi/ml). Isotope was omitted from incubations used for determination of phosphorylase *a* activity. After shaking for 60 min at 37°C in a water bath under an atmosphere of O_2/CO_2 (95:5), hormone was added and incubation continued for the times indicated before removal of samples for phosphorylase *a* activity measurement [9] or for electrophoresis of ^{32}P-labeled phosphoproteins. For the latter, 20 μl of cell suspension was rapidly mixed with 80 μl of treatment buffer containing 9.5 M urea, 2% ampholines, 2% (w/v) nonidet P40, 25 mM dithiothreitol, 10 mM EDTA, 50 mM NaF and 0.5% SDS, and immediately frozen in liquid N_2 until processed further. For one-dimensional SDS-polyacrylamide gel electrophoresis the procedure in [17] was used employing a 10% polyacrylamide separating gel. Two-dimensional gel electrophoresis was carried out as in [18]. Densitometric analysis was carried out as in [19].

3. RESULTS AND DISCUSSION

The identification of endogenous substrates for diglyceride-activated protein kinase C is a key element in understanding the role of this kinase in cellular processes. The phospholipid dependency of protein kinase C may indicate a close, functional association with cellular membranes, and distribution studies of the enzyme in liver following tissue disruption do in fact show a significant, albeit variable, proportion associated with particulate fractions [11,20]. In the present study phosphorylation of liver plasma membranes by endogenous protein kinase C has been examined. Table 1 shows the distribution of marker enzymes in this plasma membrane fraction employed. The plasma membrane fraction was substantially enriched in the plasma membrane marker, 5′-nucleotidase (54-fold relative to the original homogenate compared with values of 0.36 and 0.39 for cytochrome oxidase and NADPH cytochrome *c* reductase, respectively). Contamination of the plasma membrane fraction with mitochondria was about 7%, based on a value for cytochrome oxidase activity in isolated mitochondria of 1025 ± 73 nmol · min^{-1} · mg^{-1}. Fig.1 shows

Table 1

Enzyme distribution in purified plasma membrane fraction and liver homogenate

Fraction	Enzyme activity (nmol · min^{-1} · mg^{-1})		
	5′-Nucleotidase	Cytochrome oxidase NADPH	Cytochrome *c* reductase
Liver homogenate	5.87 ± 0.15	207 ± 6	8.13 ± 0.09
Plasma membrane	318 ± 10	74.3 ± 2.7	3.19 ± 0.53

Enzyme activities and liver fractionation were as described in section 2. Results are the mean ± SE of triplicate determinations

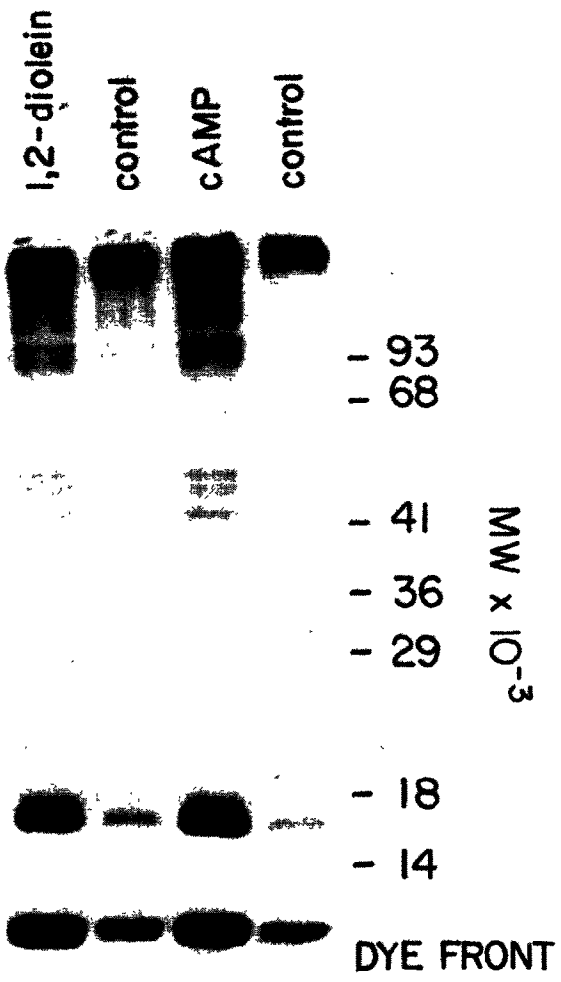

Fig. 1. Autoradiograph showing phosphorylation of rat liver plasma membranes by endogenous protein kinases. Membranes (2.25 mg/ml) were preincubated at 30°C for 2 min in 20 mM Hepes (pH 7.2) containing no further additions (control), 1,2-diolein (10 μg/ml) or cAMP (10 μM), as indicated. Reaction was initiated by addition of [γ-^{32}P]ATPMg^{2-} (100 μM ATP, 5 mM $MgCl_2$, specific radioactivity 524 mCi/mmol) and terminated after 30 s by addition of medium containing EDTA and SDS (final concentrations of 6 mM and 2%, respectively). Samples were analyzed by SDS gel electrophoresis using a 10% acrylamide separating gel.

a radioautograph depicting phosphorylation of rat liver plasma membranes by intrinsic protein kinases with [γ-^{32}P]ATP as substrate. Clearly, 1,2-diolein (10 μg/ml) and cAMP (10 μM) produced marked increases in phosphorylation of a band with M_r of about 16000. On the basis of Coomassie blue staining there were no significant differences in protein staining profile in any of the lanes indicated. In 3 different membrane preparations the stimulation of 16-kDa protein phosphorylation elicited by 1,2-diolein and cAMP was 289 ± 34% and 359 ± 74%, respectively (means ± SE). The cAMP-dependent protein kinase inhibitor [21] reduced the cAMP-stimulated phosphorylation of the 16-kDa protein to the control level, but was without effect on the stimulation elicited by 1,2-diolein (not shown). These findings, together with the observation that monoolein (10 μg/ml) did not stimulate 16-kDa protein phosphorylation, suggest that the increased 16-kDa protein phosphorylation in response to 1,2-diolein may reflect increased protein kinase C activity.

Fig.2 shows the effect of incubation of a high speed (100000 × g) liver supernatant fraction with [γ-^{32}P]ATP under conditions designed to activate endogenous protein kinases, namely protein kinase C, cAMP-dependent protein kinase and Ca^{2+}-calmodulin-dependent protein kinase(s). The upper panel (A) of fig.2 shows that cAMP appears to phosphorylate a number of proteins of M_r, 60000, 29000, 24000, 22000 and 16000. Incubation with $CaCl_2$ in the presence or absence of phosphatidylserine (panel 2B) leads to increased phosphorylation of a 93-kDa protein whilst in the presence of phosphatidylserine and $CaCl_2$, conditions which would be expected to activate protein kinase C [10,11], there is a marked increase in the phosphorylation of a 16-kDa protein. Phosphatidylserine itself produced no increase in phosphorylation in the absence of $CaCl_2$ (not shown). The lower panel (fig.2C) shows a radioautograph depicting the separation by 2-dimensional gel electrophoresis of the cytosolic proteins phosphorylated in the presence of $CaCl_2$ and phosphatidylserine. A 16-kDa protein (identified by the arrow) is clearly phosphorylated under these conditions.

The physiological relevance of these in vitro observations concerning phosphorylation of a 16-kDa protein in soluble and plasma membrane fractions of liver was examined in studies with isolated hepatocytes prelabeled with $^{32}P_i$ followed by exposure to vasopressin for various times and subsequent analysis of changes in ^{32}P protein labeling by 2-dimensional gel electrophoresis [18]. Fig.3

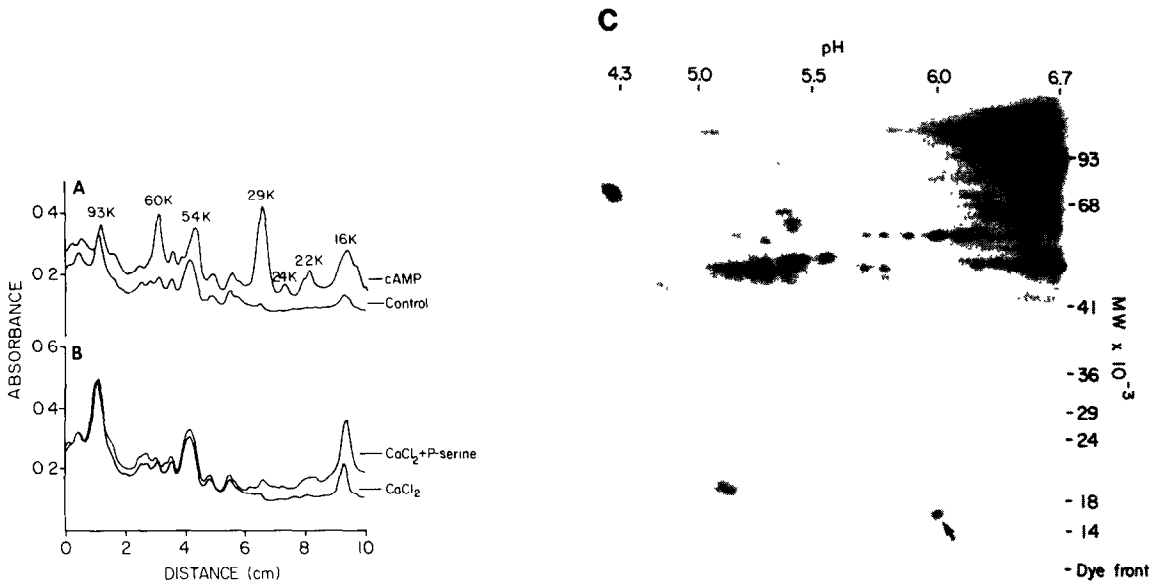

Fig. 2. cAMP, Ca^{2+} and protein kinase C-mediated phosphorylation of liver cytosolic proteins. A high speed supernatant (100 000 × *g*) was prepared from rat liver by homogenization in 0.25 M sucrose, 25 mM Tris-HCl (pH 7.5) 2 mM EDTA and 10 mM 2-mercaptoethanol. Phosphorylation of cytosolic proteins was carried out by incubation for 2 min at 30°C with 50 μM [γ-^{32}P]ATP (specific radioactivity 750 mCi/mmol) in medium containing 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.25 mM EGTA and other additions where indicated; control, none; cAMP, 10 μM; $CaCl_2$, 0.5 mM; $CaCl_2$ + P-serine, 0.5 mM $CaCl_2$ and 25 μg/ml L-α-phosphatidylserine, respectively. Reaction was terminated by addition of medium containing EDTA and SDS (final concentrations of 2 mM and 2%, respectively) and samples were analyzed by one-dimensional SDS-gel electrophoresis (panels A,B). Panel C shows a radioautograph depicting the 2-dimensional gel electrophoretic separation of the cytosolic proteins phosphorylated in the presence of $CaCl_2$ and phosphatidylserine. In this case aliquots of the incubation medium were mixed with lysis buffer (9.5 M urea, 2% w/v nonidet P40, 2% ampholines, 25 mM dithiothreitol, 10 mM EDTA, 50 mM NaF and 0.5% SDS and frozen in liquid N_2. Following 2-dimensional gel electrophoresis the dried gels were exposed to Dupont Cronex 4 X-ray film for 3 weeks.

shows that phosphorylase is almost maximally activated by vasopressin (10^{-8} M) after 30 s. For analysis of the corresponding changes in ^{32}P phosphoprotein labeling over a similar time period, the cells were rapidly mixed with medium containing high concentrations of urea, SDS, NaF and EDTA and immediately frozen in liquid N_2. Comparison of panels A and B of fig.4, i.e., before hormone (10^{-8} M vasopressin) and 1 min thereafter, shows a marked hormone-induced increase in the phosphorylation of a 16-kDa protein having a p*I* value of about 5.6. Vasopressin-induced changes in the phosphorylation state of several other proteins can also be observed, notably a protein of M_r 49 000 which appears as a series of 6 or 7 spots (p*I* 4.8–5.3) and a protein M_r 61 000 (p*I* 6.5) previously described in [22]. At other times following vasopressin stimulation (panels C, D, E, F) a second, fainter spot corresponding to a slightly less acidic species of 16-kDa protein (p*I* ~5.8) can also be observed. The relationship of this 16-kDa phosphoprotein species to the major phosphorylated form (panel B), remains to be ascertained. As shown in panel C, increased phosphorylation of the 16-kDa protein (p*I* 5.6) can be detected as early as 15 s after vasopressin treatment, appears to be maximal at 30 s and 2 min after vasopressin treatment (panels D and E, respectively) and by 5 min appears to have undergone substantial dephosphorylation (panel F). Interestingly, as can also be observed from panels E and F, there appears to be concomitant dephosphorylation of a 22-kDa protein (p*I* ~ 6.2), whereas over a similar period of time phosphoryl-

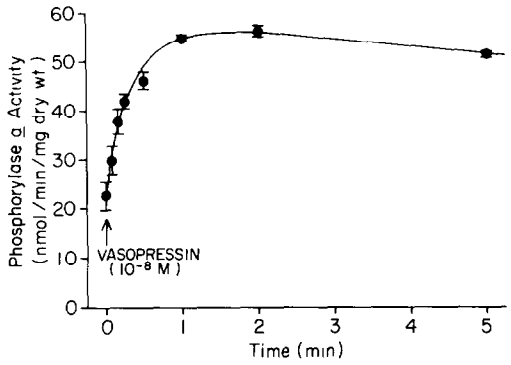

Fig. 3. Time course of vasopressin-induced phosphorylase activation in isolated hepatocytes. Hepatocytes (5 mg dry wt/ml) were incubated for 60 min at 37°C in a modified Krebs bicarbonate buffer as described in section 2. For phosphorylase assay, aliquots were removed at the times indicated into medium containing 100 mM Mops, 150 mM NaF, 20 mM EDTA, 400 mM sucrose, 5 mM dithiothreitol, 0.03% Triton X-100 (pH 7.0) and immediately frozen in liquid N_2. Results are means ± SE for 3 incubation flasks.

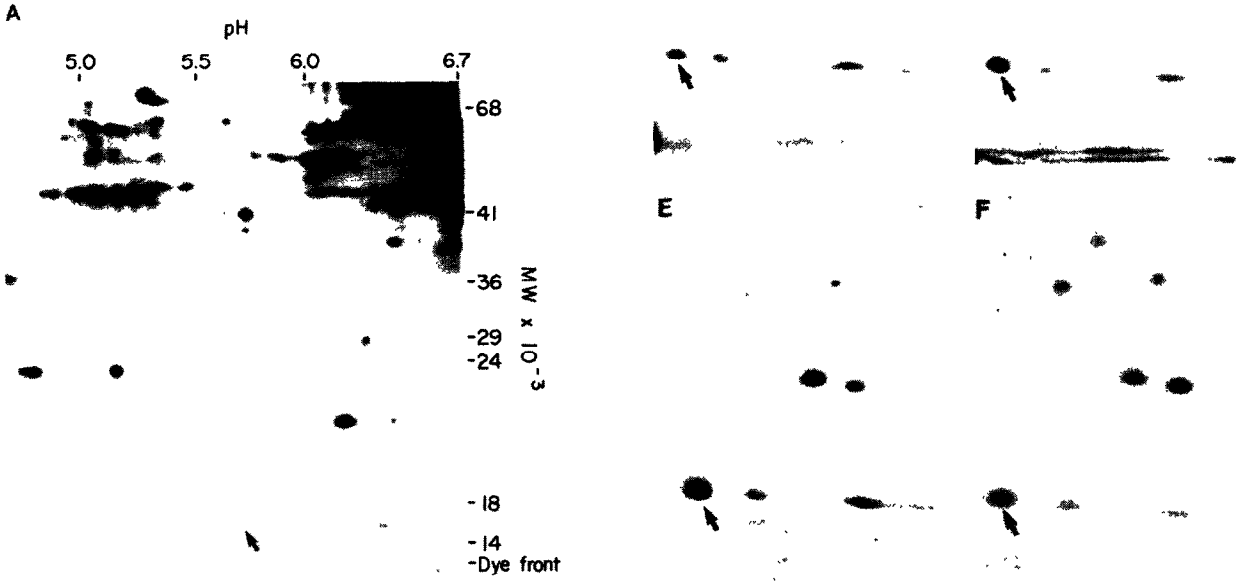

Fig. 4. Radioautographs depicting the time course of phosphorylation of proteins in ^{32}P-labeled hepatocytes in response to vasopressin. Hepatocytes were preincubated for 60 min at 37°C as described in the legend to fig.3 with the further inclusion of ^{32}Pi (1 mCi/ml). Following addition of vasopressin (10^{-8} M) aliquots of the cell suspension were rapidly mixed with lysis buffer and thereafter processed as described in the legend to fig.2. Panels represent; A, control immediately prior to hormone addition; B, 1 min after vasopressin addition; C, D, E, F, 15 s, 30 s, 2 min and 5 min after vasopressin. Relevant areas of the original radioautographs are depicted.

ase remains almost maximally activated (cf. fig.3). Such observations which presumably reflect differential kinase and/or phosphatase activities, clearly emphasize the importance of analyzing kinetic changes of ^{32}P-phosphoprotein labeling patterns in attempting to understand further the complexities of cellular regulation by phosphorylation–dephosphorylation.

In summary, our study indicates that a 16-kDa protein can be phosphorylated in vitro by protein kinase C, and such a mechanism could account for the observed changes in 16-kDa protein phosphorylation in the intact hepatocyte. Although the functional significance of this protein is as yet unknown, the present findings suggest that it could be part of the coordinated mechanism associated with hormonal regulation of cellular Ca^{2+} fluxes. Studies are in progress to determine whether phosphorylation of the 16-kDa protein is involved in the regulation of liver plasma membrane Ca^{2+} fluxes.

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